Developmental Biology **248,** 157–169 (2002)
doi:10.1006/dbio.2002.0713

Vestibular Morphogenesis in the Mouse Embryo through a BMP4-Mediated Pathway

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In the mouse embryo, *Dlx5* is expressed in the otic placode and vesicle, and later in the semicircular canals of the inner ear. In mice homozygous for a null *Dlx5/LacZ* allele, a severe dysmorphogenesis of the vestibular region is observed, characterized by the absence of semicircular canals and the shortening of the endolymphatic duct. Minor defects are observed in the cochlea, although *Dlx5* is not expressed in this region. *Cristae* formation is severely impaired; however, sensory epithelial cells, recognized by calretinin immunostaining, are present in the vestibular epithelium of *Dlx5*$^{-/-}$ mice. The *maculae* of utricle and saccule are present but cells appear sparse and misplaced. The abnormal morphogenesis of the semicircular canals is accompanied by an altered distribution of proliferating and apoptotic cells. In the *Dlx5*$^{-/-}$ embryos, no changes in expression of *Nkx5.1(Hmx3), Pax2,* and *Lfng* have been seen, while expression of *bone morphogenetic protein-4 (Bmp4)* was drastically reduced. Notably, BMP4 has been shown to play a fundamental role in vestibular morphogenesis of the chick embryo. We propose that development of the semicircular canals and the vestibular inner ear requires the independent control of several homeobox genes, which appear to exert their function via tight regulation of *BPM4* expression and the regional organization of cell differentiation, proliferation, and apoptosis.

***Key Words: Dlx*; homeobox gene; transcription factor; bone morphogenetic protein-4; inner ear; apoptosis.**

INTRODUCTION

Development of the inner ear represents a fascinating morphogenetic program with still unresolved molecular basis. The dorsal portion of the otocyst gives rise to the vestibular organ, which consists of three precisely shaped and oriented semicircular canals, utriculus and sacculus, while the medial–ventral region gives rise to the cochlea (Li *et al.*, 1978; Torres and Giraldez, 1998). The first critical steps of semicircular canal morphogenesis take place between 11.5 and 13.5 dpc, long after general otocyst patterns have been established, and include the growth of canal pockets, formation and fusion of the canal plates, and their subsequent disappearance (Martin and Swanson, 1993). Little is known about the cellular and molecular mechanisms underlying morphogenesis and pattern formation in the inner ear (Fekete, 1996, 1999; Represa *et al.*, 2000). In recent years, a number of genes expressed in the otocyst have been isolated (Torres and Giraldez, 1998). Their gene products can be classified as transcription factors, diffusible molecules, receptors, and adhesion proteins. The earliest expressed genes in the otic placode include the *distal-less* related (*Dlx3*), the *muscle segment homeobox*-related (*Msx1*), *NK*-related, and *LIM*-domain. As postulated by Fekete (1996) and Ryan (1997), the establishment of precisely spaced expression domains of transcription factors within the otocyst lays down the molecular blueprint for

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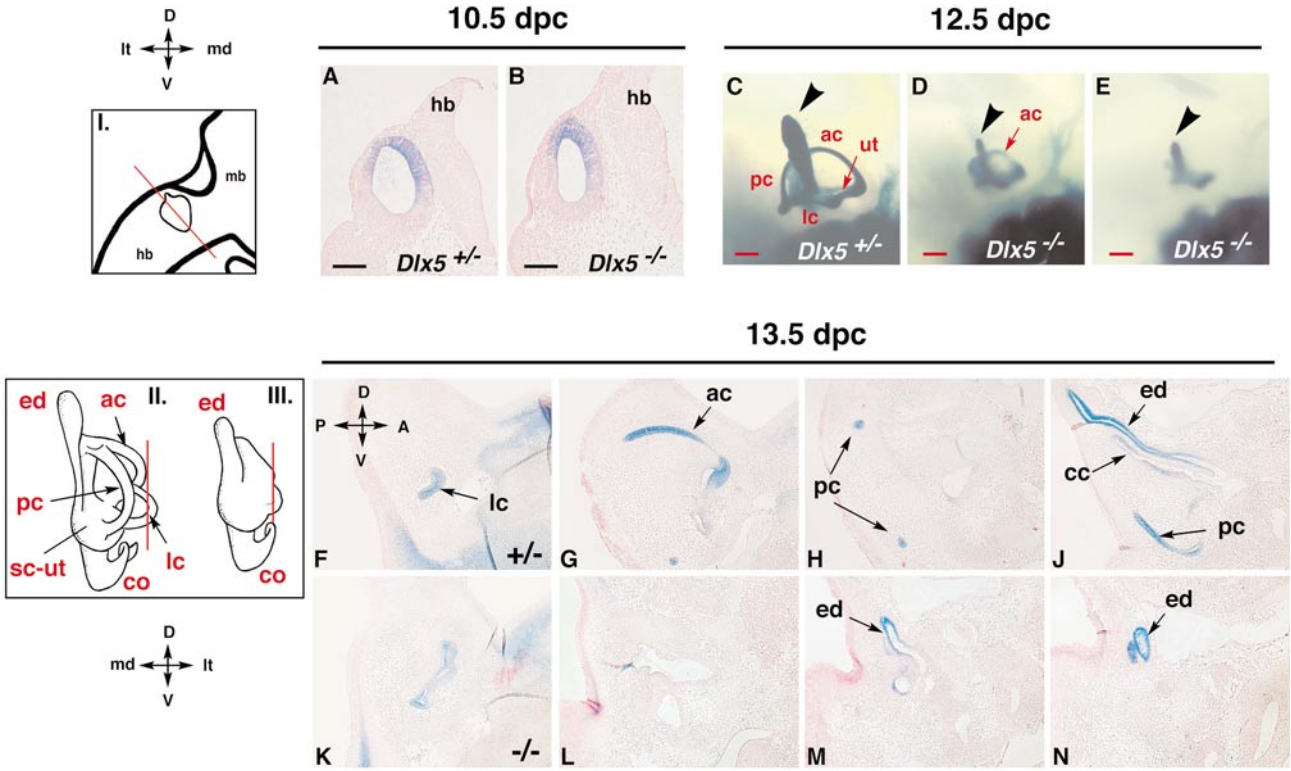


FIG. 1. Inner ear of *Dlx5*$^{-/-}$ embryos. (A, B) Frontal sections of the otocyst of heterozygous (A) and homozygous (B) 10.5-dpc *Dlx5*$^{-/-}$ embryos, whole-mount stained with X-gal, counterstained with hematoxylin. Orientation and section plane are shown (I). (C–E) Whole-mount X-gal staining of the otocyst of 12.5-dpc embryos, in lateral view. The semicircular canals of heterozygous (C) and homozygous (D, E) embryos are visible. (D, E) A "mild" and a "severe" phenotype, respectively. Black arrowheads indicate the endolymphatic duct. For (C–E), the same magnification was used. (F–N) Serial histology of the inner ear of *Dlx5* heterozygous (F–J) and homozygous (K–N) 13.5-dpc embryos, stained with X-gal. The orientation is shown (F). Diagrams of a normal (II) and a *Dlx5* null (III) inner ear are reported, to show the orientation and section planes (red line). ac, anterior canal; co, cochlea; cc, *crus communis* of the semicircular ducts; ed, endolymphatic duct; hb, hindbrain; lc, lateral canal; mb, midbrain; pc, posterior canal; sc-ut, saccule–utricle region; ut, utricle. Scale bars in (A–E), 100 μm.

the subsequent morphogenetic program. For example the dorsolateral aspect of the vesicle (precursor of the semicircular and endolymphatic ducts) expresses *Nkx5.1(Hmx3)*, *Dlx3*, *sox9*, *Dlx5*, and others, while the medial and ventral aspects (precursor of the cochlea and the sensory epithelium) express *Pax2*. The complementary expression of transcription factors in the vestibular vs the cochlear portions of the vesicles raises hopes that one could define the molecular regulation responsible for early otocyst patterning by using targeted gene disruption in the mouse. For example, *Pax2* is expressed only in the acoustic region of the otocyst (Herbrand *et al.*, 1998) and its inactivation leads to agenesis of the cochlea (Torres *et al.*, 1996). *Nkx5.1* is expressed in a complementary way in the vestibular compartment of the otocyst and controls the formation of the semicircular canals (Rinkwitz-Brandt *et al.*, 1996; Herbrand *et al.*, 1998; Hadrys *et al.*, 1998; Wang *et al.*, 1998).

The development of the three-dimensional organization, regional specialization, and cell differentiation within the inner is partly the result of a precise spatiotemporal regulation of basic cellular events, such as proliferation and apoptosis. Secreted diffusable molecules are likely to play a major role in this regulation. Of particular interest is the proposed function of members of the bone morphogenetic protein (BMP) family of peptides, and in particular BMP4, as inner ear morphogens. Expression of *Bmp4* in the mouse, chick, and *Xenopus* embryos is found in the prospective sensory area of the otic epithelium, with species-specific differences (Oh *et al.*, 1996; Morsli *et al.*, 1998; Cole *et al.*, 2000). Functional studies in the chick embryo in which *noggin* was used to sequester endogenous BMP4 clearly point to a role for BMP4 in the growth and morphogenesis of the vestibular region of the inner ear (Chang *et al.*, 1999; Gerlach *et al.*, 2000). Regrettably, *Bmp4* null mice die at early stages of embryonic development and cannot be studied for inner ear defects (Winnier *et al.*, 1995).

Dlx genes encode for *distal-less*-related transcription factors with similar *in vitro* DNA-binding properties (Simeone

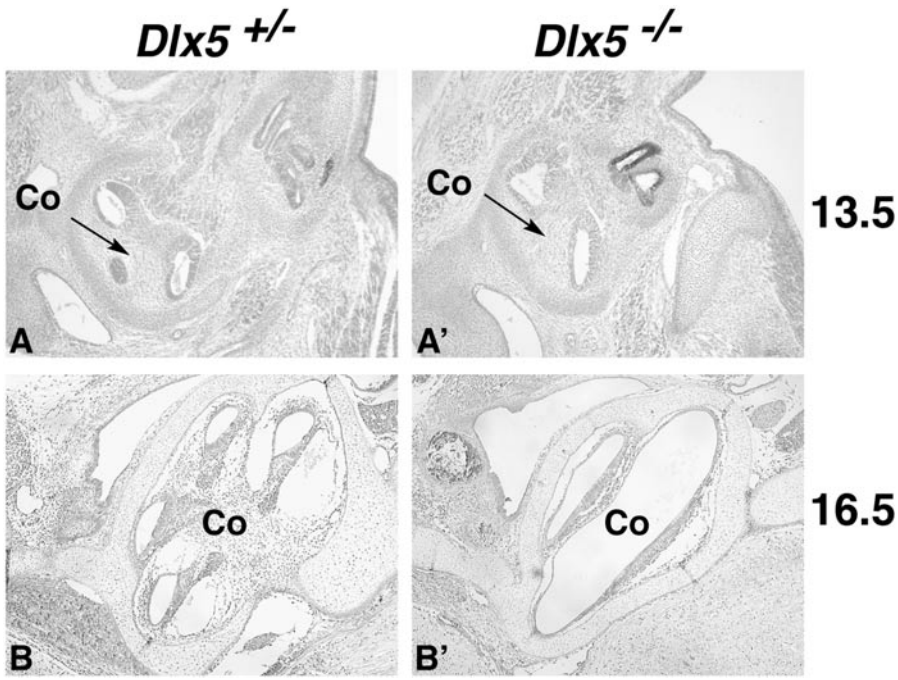


FIG. 2. Histology of the cochlea in $Dlx5^{-/-}$ embryos. Sagittal sections of normal (A, B) and $Dlx5^{-/-}$ (A′, B′) embryos are compared at 13.5 (A, A′) and at 16.5 (B, B′) dpc of development. co, cochlea.

et al., 1994; Liu *et al.*, 1997; Zhang *et al.*, 1997; Merlo *et al.*, 2000). Several members of the *Dlx* gene family are expressed in the otic vesicle (Robinson and Mahon, 1994; Qiu *et al.*, 1997; Acampora *et al.*, 1999). The *Dlx5* gene, in particular, is expressed in the otic placode and vesicle and is rapidly restricted to a dorsal region, precursor of the vestibular organ. We (Acampora *et al.*, 1999) and others (Depew *et al.*, 1999) have generated mice null for *Dlx5*, in which a dysmorphogenesis of the inner ear was noted. In this paper, we characterize the inner ear defect in the *Dlx5* null mice. The similarity of inner ear defects observed in *Dlx5* with those reported in the chick embryo following *noggin*-induced BMP4 depletion (Chang *et al.*, 1999; Gerlach *et al.*, 2000) prompted us to examine *Bmp4* expression in these mutant mice. The results suggest that BMP4 may function as a *Dlx5*-dependent inner ear morphogen and that its altered expression may lead to altered distribution of early apoptotic cells.

MATERIALS AND METHODS

Generation of Dlx5 Null Mice

Mice with a targeted disruption of the *Dlx5* gene have been reported (Acampora *et al.*, 1999; Depew *et al.*, 1999). We have generated a null *Dlx5* allele by replacing its Start codon and first two exons with the *LacZ* reporter. This modification provides a marker for *Dlx5*-expressing cell lineages and enabled us to easily compare the heterozygous with the homozygous mutant embryos. X-gal staining of heterozygous embryos recapitulates the known pattern of *Dlx5* expression (Acampora *et al.*, 1999, Merlo *et al.*, 2000; G.R.M., unpublished data). Mice with a targeted inactivation of *Nkx5.1(Hmx3)* have been reported (Hadrys *et al.*, 1998). Since no defect has ever been observed in the heterozygous animals, in this article we will refer to either wild-type (+/+) or heterozygous (+/−) mice as "normal." Embryos were staged considering the day of the plug as 0.5 dpc. Genotypes were determined by PCR amplification as described (Acampora *et al.*, 1999).

Histological and Histochemical Techniques

For the detection of *LacZ* reporter expression, postimplantation embryos were collected, fixed for 30 min (8.5–9.5 dpc) or 2 h (10.5–11.5 dpc) in 4% paraformaldehyde (PAF) in 0.1 M Phosphate Buffer (PB), washed in PB–Saline (PBS), and stained with X-gal as described (Acampora *et al.*, 1999). Stained embryos were washed in PBS, photographed, and either clarified in a 2:1 benzyl-benzoate/benzyl alcohol mix or embedded in paraffin by using standard protocols, and then sectioned at 6 μm thickness. Mallory's thrichromic staining was carried out as indicated in histology manuals. Immunohistochemistry was carried out on paraffin sections by using primary rabbit antisera or mouse monoclonal antibodies, revealed with, respectively, the EnVision+ peroxidase-conjugated goat anti-rabbit or the ARK kit (Dako), according to the manufacturer's specifications, and visualized with DAB. The antibodies used were: mouse monoclonal anti-bromo-deoxy-uridine (BrdU) (1:50; Dako), rabbit anti-calretinin (1:1000; Chemicon International), and rabbit anti-β-gal (1:5000; Cappel–ICN Biomedicals). Whole-mount immunohistochemistry on 10.5-dpc embryos was

performed as described (Schneider-Maunoury *et al.*, 1993) by using the 2H3 anti-neurofilaments monoclonal antibody (Developmental Studies Hybridoma Bank, Univ. of Iowa, Dept. Biological Sciences, Iowa City), diluted 1:5000. After color development, the head ectoderm and some mesoderm were removed to expose the nerves and ganglia. A total of 12 embryos were analyzed and photographed. The whole-mount staining with 2H3 antibody was specifically used to reveal the trajectory and extension of cranial nerves.

Cell Proliferation and Apoptosis

Cell proliferation was assayed by BrdU incorporation followed by immunohistochemistry with anti-BrdU antibody on serial sections. Pregnant females (10.5 and 11.5 dpc) were injected three times intraperitoneally with BrdU (Sigma, 30 μg/g of body weight) at 2-h intervals and sacrificed 10 h after the first injection. Embryos were fixed 4 h in 4% PAF, rinsed in PBS, paraffin embedded, and sectioned at 6 μm thickness. Serial sections of the otocyst were then stained with anti-BrdU antibody and examined. Two normal and three mutant embryos were analyzed. Apoptotic cells were detected on a complete series of 6-μm paraffin sections of the inner ear region of 11.5-dpc embryos, using the TUNEL method (Roche), according to the manufacturer's instruction. Embryos were collected, fixed, and paraffin embedded as for normal histology. Three normal and four mutant embryos were analyzed. Controls for the TUNEL procedure were mouse mammary glands during lactation (negative control) or involution (positive control).

Whole-Mount in Situ Hybridization

In situ RNA:RNA hybridization with specific probes was carried out on 9.5-dpc normal and homozygous *Dlx5/LacZ* mouse embryos. Synthesis of the DIG-labeled RNA probes was carried out by *in vitro* transcription of linearized plasmid vectors containing the appropriate cDNA sequence (Promega). Hybridization was carried out as described (Wilkinson, 1992) and detection was done with anti-DIG FAb and BCIP/NBT development. At least three embryos of each genotype were analyzed with each probe. Selected embryos were finally embedded in gelatin, thick-sectioned (100 μm) using a Leika Vibratome, and photographed. The probes for *Nkx5.1(Hmx3)* and *Pax2* have been reported (Rinkwitz-Brandt *et al.*, 1996; Hadrys *et al.*, 1998). The probes for murine *Bmp4* and *Lunatic Fringe* (*Lfng*) were a gift from Dr. Doris Wu (Rockville, MD) (Morsli *et al.*, 1998). The probe for *Otx1* was a 396-nucleotide *Sac*I–*Pst*I fragment (Simeone *et al.*, 1993).

RESULTS

In Dlx5 Mutant Embryos, the Semicircular Ducts Fail to Form

The targeted insertion of the *LacZ* reporter into the *Dlx5* locus provides a tool for detecting *Dlx5* gene transcription in both heterozygous and homozygous *Dlx5* mutant embryos. β-Gal activity is detected as early as 8.0–8.5 dpc in the otic placode, and later in the otic vesicle (Acampora *et al.*, 1999). Beginning at about 10 dpc, β-gal staining localizes to a dorsal region of the otocyst, the presumptive area of the vestibular organ (Figs. 1A and 1B). In all subsequent stages, *Dlx5/LacZ* is found exclusively in the epithelium of the membranous labyrinth, confined to the semicircular canals and their insertion points on the utricle, and the endolymphatic duct (Fig. 1C). Expression of *Dlx5/LacZ* is also found in the presumptive sensory territories, the developing *cristae* (Fig. 1C; and data not shown). Expression is minimal or absent in the utricle, absent in the saccule and in the cochlea, their sensory organs, and in the cochlear–vestibular ganglion (CVG). We have therefore restricted the following comparative expression analysis of control and *Dlx5* null embryos to the vestibular portion of the inner ear.

In $Dlx5^{-/-}$ embryos, expression of *Dlx5/LacZ* is localized to a dorsal domain of the otocyst very similar to that seen in the heterozygous embryos, indicating that the *Dlx5*-expressing cell lineage is present in the *Dlx5* null embryos, in its normal position (Figs. 1A–1E). No morphological difference was observed at earlier stages (9.5–10.5 dpc). A dysmorphogenesis of the otocyst begins to appear at 11.5 dpc, but becomes more evident at 12.5 dpc. The defect consists of a severe hypoplasia of the primordia of the semicircular canals and a failure of elongation of the endolymphatic duct (Figs. 1C–1E; Acampora *et al.*, 1999), with a varying degree of severity. In mutant 12.5-dpc embryos exhibiting a milder phenotype (Fig. 1D), the hypoplasia is less pronounced, the epithelium precursor of the canals folds (often the lateral canal can be identified), but canal plates never form properly, morphogenesis does not proceed further, and no fusion of the central plates occurs. The anterior and posterior canals can rarely be recognized, being replaced by a single compartment. The endolymphatic duct forms but remains shorter and broader. Finally, the utricle is enlarged and deformed, while the saccule forms normally. In more severe phenotypes (Fig. 1E), no canal-like structure can be recognized altogether. The entire canal region appears to be reduced to a single vesicle lined by a thinner single-layered epithelium, the endolymphatic duct remains rudimentary, the utricle is expanded and deformed, and the saccule appears only slightly affected.

To further characterize the morphology of mutant inner ears, serial sections of X-gal-stained normal and $Dlx5^{-/-}$ embryos were compared (Figs. 1F–1N). At 13.5 dpc, no overt canal structure is observed, being apparently replaced by an X-gal-positive canal-like structure resembling a fusion of the lateral canal with the joining point of the lateral and anterior canals onto the anterior utricle. Remains of the semicircular ducts are also seen in a region that corresponds with the *crus communis* and part of the anterior and superior canals. The endolymphatic duct can be easily identified and appears shorter and broader (Figs. 1M and 1N). Finally, we examined the cochlear region of the inner ear of normal and $Dlx5^{-/-}$ embryos at 13.5 and 16.5 dpc, by serial histology (Fig. 2). We have previously shown that the cartilage capsule surrounding the acoustic region of the inner ear of newborn $Dlx5^{-/-}$ mice does not show any obvious abnormality (Acampora *et al.*, 1999), except for a noticeable rotation toward the midline. At 13.5 dpc, the cochlea of $Dlx5^{-/-}$ embryos extends normally in a medial–

ventral direction but shows a slightly retarded growth (Figs. 2A and 2A′). At 16.5 dpc, the incomplete growth of the cochlea becomes more evident (Figs. 2B and 2B′), and is accompanied by a rotation of the cartilage capsule toward the midline. These data are consistent with the results presented in Depew *et al.* (1999), showing that the cochlea of *Dlx5*$^{-/-}$ mice does not attain a proper degree of coiling and that the number of turns is reduced. No expression of *Dlx5* has been observed in the mouse cochlea at any stage (Fig. 1; Acampora *et al.*, 1999; Depew *et al.*, 1999; G.R.M., unpublished observations). Thus, the cochlear defects cannot be a direct result of *Dlx5* disruption and were not further investigated.

In summary, the histological defects of *Dlx5*$^{-/-}$ inner ear consist of a severe size reduction and lack of morphogenesis of the semicircular canals, the failure of endolymphatic duct elongation, the fusion of the utricle with the rudiments of the canals into a single compartment, and a milder dysmorphogenesis of the cochlea.

Analysis of the Sensory and Neural Components of the Inner Ear

Dlx5/LacZ is expressed in areas of the developing semicircular ducts that include the presumptive sensory organs. We have investigated sensory development in *Dlx5*$^{-/-}$ animals. No histologically recognizable *ampullae* or *cristae* were observed in *Dlx5*$^{-/-}$ mice at any stage of development and at birth (Fig. 3; and data not shown). Patches of a thicker epithelium—corresponding to the *maculae*—are present in the utricle and saccule of the mutant mice. In order to clarify whether these epithelial areas of the mutant inner ear correspond indeed to the *maculae*, and to monitor sensory cell differentiation in the rudimentary semicircular canals, serial sections of 16.5-dpc normal and mutant embryos were immunostained for calretinin, a marker for all postmitotic sensory cells of the inner ear (Zheng and Gao, 1997). In the wild-type embryo at this stage, calretinin immunoreactivity is found in all the developing *cristae* (Figs. 3A, 3B, and 3E) and *maculae* (Figs. 3C and 3D), but not in the acoustic organ. In the *Dlx5*$^{-/-}$ embryos, a patch of thickened epithelium, including a small cluster of calretinin-immunoreactive cells, is observed in a region roughly corresponding to a *crista* (Fig. 3J). Other patches of thickened calretinin-positive epithelium corresponding to the *maculae* of the utricle and saccule were seen in nearly normal positions (Figs. 3G and 3H). In these areas, however, the general appearance of the sensory epithelium was not normal: cells were sparsely distributed and disorganized, and the calretinin signal intensity was diminished, indicating a possible maturation delay. Finally, in the *Dlx5*$^{-/-}$ inner ear, groups of calretinin-immunoreactive cells were seen in ectopic positions (Figs. 3F and 3I), an indication of altered positional information. In conclusion, in *Dlx5*$^{-/-}$ embryos, the sensory epithelial cell lineage is generally able to initiate differentiation but fails, to a large extent, to organize in the *cristae*, the sensory cells of the *maculae* appear sparse and disorganized, and sensory cells can be found ectopically located.

To rule out that a generalized patterning defect involves the entire otic region, the cranial nerves and ganglia of *Dlx5*$^{-/-}$ embryos were whole-mount immunostained for neurofilaments with the 2H3 antibody. This technique is superior to serial histology in that the exact position, trajectory, and length of each nerve can be accurately compared. Several normal and homozygous mutant embryos at 10.5 dpc were carefully examined, but no difference could be observed in the pattern of innervation in the otic region (Fig. 4).

We also examined the morphology of CVG in normal and *Dlx5*$^{-/-}$ embryos at 11.5 dpc and at birth. No obvious difference was observed in the *Dlx5* null vs normal ones (data not shown). In addition, the CVG of normal and *Dlx5*$^{-/-}$ embryos at 11.5 dpc shows a comparable number and location of apoptotic cells and no BrdU positive cells (data not shown). These findings are consistent with the lack of *Dlx5/LacZ* expression in the ganglion presumptive region of the otocyst and in the CVG.

Expression of Nkx5.1, Pax2, Otx1, and Lfng in Dlx5 Null Embryos

The failure of proper canal formation and the distorted organization of the vestibular sensory epithelium suggest patterning defects in the developing otic epithelium. Therefore, gene expression patterns were compared in *Dlx5* null and control embryos by whole-mount *in situ* hybridization with antisense RNA probes. At first, two region-specific markers that broadly identify the vestibular (*Nkx5.1*) (Rinkwitz-Brandt *et al.*, 1996; Hadrys *et al.*, 1998) or the cochlear (*Pax2*) (Herbrand *et al.*, 1998) region within the otocyst were used, respectively. In addition *Otx1* expression, normally found in the presumptive lateral canal region, its *ampulla*, and in the utricle (Morsli *et al.*, 1999), was analyzed. No evident changes in the expression territory of any of these markers were observed in the mutant embryos compared with the normal ones (Figs. 5A–5D; and data not shown), with the possible exception of *Pax2* whose expression territory appears minimally expanded dorsally. Thus, the general patterning and subdivision of the otic vesicle into a dorsal vestibular-forming and a ventral cochlea-forming component is maintained in the *Dlx5* null embryos.

Subsequently, two additional hybridization markers encoding potential otic morphogenes, *Lfng* and *Bmp4*, were used. These molecules may be indicative for changes in the identity and fate of the sensory patches within the otic epithelium (Cole *et al.*, 2000), and also for more general morphogenetic changes in the inner ear. *Bmp4* expression will be described below. *Lfng* mRNA is normally detected in 9.5- to 12.5-dpc embryos in anterior–ventral territories of the otocyst, the precursors of the sensory organ of the cochlea, and in the *maculae* of the utricle and saccule. At

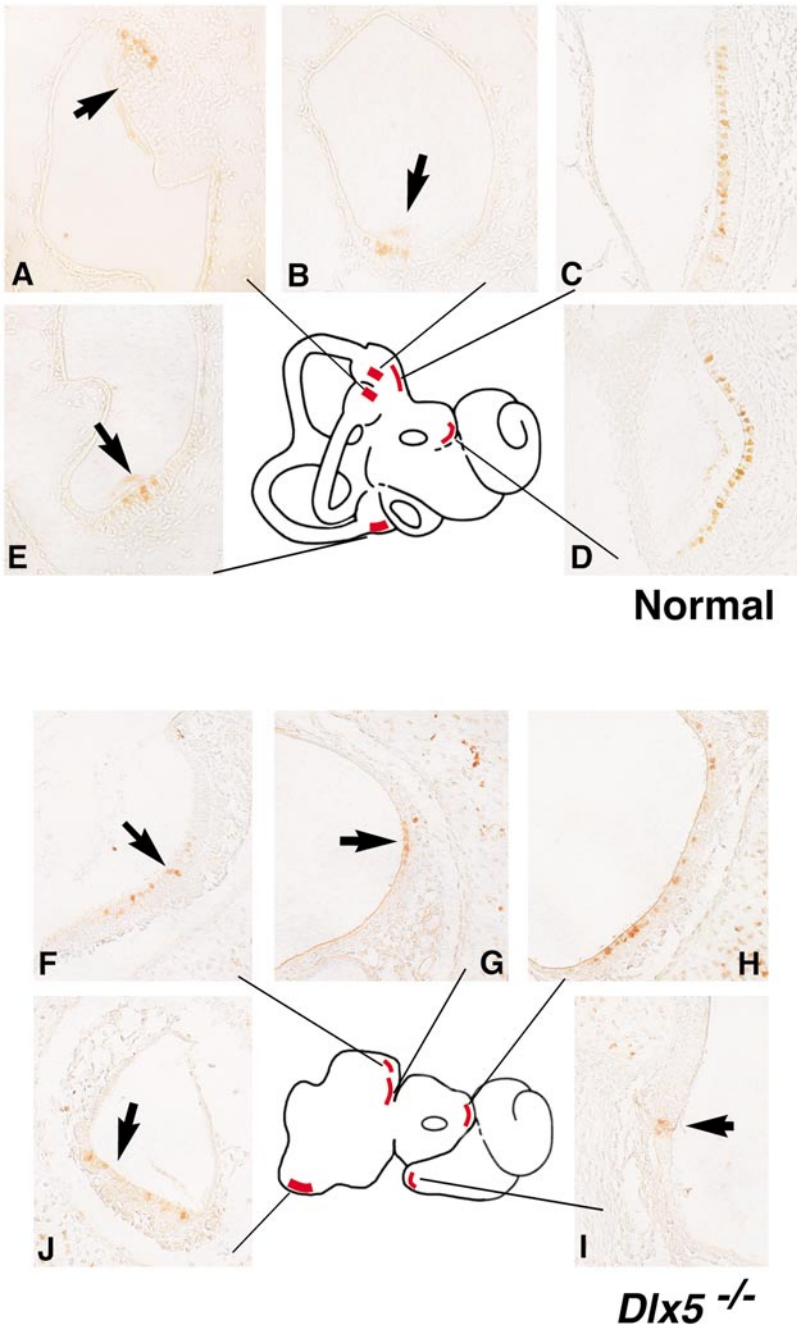


FIG. 3. Sensory areas of the inner ear in *Dlx5*$^{-/-}$ embryos. Calretinin immunostaining of serial longitudinal sections of normal (A–E) and *Dlx5*$^{-/-}$ (F–I) 16.5-dpc embryo inner ears. Two mutant embryos were analyzed. Patches of positive cells (black arrows) reveal the presence and location of differentiating sensory cells, corresponding to the known inner ear sensory organs. The location of the calretinin-positive clusters in the normal and the *Dlx5*$^{-/-}$ inner ear are reported schematically on the central diagrams (red areas).

later stages (from 13.5 dpc onward), *Lfng* mRNA is found also in the developing *cristae* (Morsli *et al.*, 1998). Four normal and three mutant 10.5-dpc embryos were analyzed by *in situ* hybridization with *Lfng*, followed by serial sectioning. No significant differences in the distribution or the intensity of the signal were observed (Figs. 5E and 5F), consistent with the restricted expression of *Dlx5* in the semicircular canals and the endolymphatic duct.

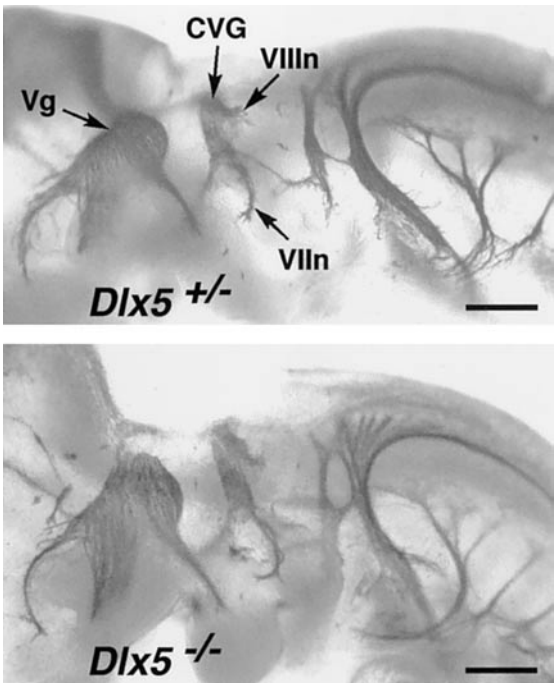


FIG. 4. Cranial nerves and ganglia of the otic region of $Dlx5^{-/-}$ embryos. A normal (top) and a homozygous (bottom) 10.5-dpc embryo stained whole mount with anti-neurofilament antibody are shown. Note that the acustic nerve (VIIIn) and the cochlear–vestibular ganglion (CVG) appear normal in the mutant animal. CVG, cochlear–vestibular ganglion; Vg, trigeminal ganglion; VIIn, facial nerve; VIIIn, acustic–vestibular nerve. Scale bars, 200 μm.

Bmp4 Expression in the Otocyst of Dlx5 and Nkx5.1 Null Embryos

Expression of *Bmp4* mRNA has been accurately mapped in the otocyst of the mouse embryo (Morsli *et al.*, 1998), in which it serves as a marker of the presumptive *cristae*. We have analyzed normal and homozygous mutant *Dlx5* and *Nkx5.1* embryos at 10.5 dpc, for expression of *Bmp4* in the otic vesicle, by whole-mount *in situ* hybridization followed by vibratome serial sectioning. The *Nkx5.1* null mutant mice show abnormal vestibular development, characterized by the absence of the posterior and lateral semicircular canals, while the anterior canal is retained in part (Hadrys *et al.*, 1998). In spite of the phenotypic similarity of the inner ear defects in *Dlx5* and *Nkx5.1* mutant embryos, these two genes are unlikely to participate in a common transcriptional cascade (Acampora *et al.*, 1999). For this reason, the otocyst of $Nkx5.1^{-/-}$ embryos can serve as a control for an independent pathway of genetic regulation of vestibular morphogenesis. In normal embryos at 10.5 dpc, *Bmp4* expression is found in two regions: an anterior domain, precursor of the superior and lateral *cristae*, and a posterior domain, precursor of the posterior *crista* (Figs. 5H, 5J, 5J′, and 5L) (Morsli *et al.*, 1998). A total of four 10.5-dpc $Dlx5^{-/-}$ embryos were examined. In three of them, *Bmp4* mRNA was totally undetectable, while in the fourth embryo, *Bmp4* signal was still present but at very low levels compared with normal embryos (Figs. 5I, 5K, and 5K′). In the latter case, the weak expression signal was diffused and occupied a region corresponding to the posterior domain of the normal otocyst (Fig. 5K′). A total of four 10.5-dpc $Nkx5.1^{-/-}$ embryos were analyzed. Expression of *Bmp4* mRNA in the $Nkx5.1^{-/-}$ was strongly reduced, but never completely absent (Figs. 5M and 5N). In $Nkx5.1^{-/-}$ embryos, the hybridization signal was rather diffused and extended to an anterior otocyst region (Fig. 5N). In addition, ectopic *Bmp4* signals appeared in a medial–posterior region and in the primordium of the cochlea (Fig. 5N), indicating a loss of positional information. Thus, in both the *Nkx5.1* and the *Dlx5* null mice, *Bmp4* expression is reduced and misregulated; however, these changes in *Bmp4* expression appear different and cannot be compared between the two mutants, in spite of the similarity in their vestibular phenotype.

Distribution of Proliferating and Apoptotic Cells in Dlx5 Null Mice

Immunohistochemical detection of BrdU-positive cells in embryos exposed to BrDU is the method of choice for estimating the number and location of cells in the S-phase of the cell cycle. We compared the distribution and density of BrdU-positive cells in the vestibular portion of the inner ear of 11.5-dpc *Dlx5* null embryos. In normal embryos, regions with a high density of BrdU-positive nuclei, indicating increased proliferation of the otic epithelium, are located on the edges of the developing canal plates (Figs. 6A and 6B), at the tip and base of the endolymphatic duct, and in restricted areas of the saccule–utricle domain (Figs. 6B–6D). In mutant embryos, the overall number of BrdU-positive cells is similar to that of normal embryos; however, their regional distribution is abnormal: BrdU-positive cells appear rather uniformly distributed and no areas of higher BrdU density can be distinguished in the canal-forming region of the otocyst (Figs. 6E and 6F). Only few higher density areas are observed in the saccule–utricle region (Figs. 6F and 6G); their position does not correspond, however, to that of normal foci of increased proliferation. In the epithelium precursor of the cochlea, the distribution of BrdU-labeled nuclei appears similar between the normal and the mutant embryos (Figs. 6D and 6H). We also examined the distribution of BrdU-labeled cells in the peri-otic mesenchyme, to establish weather altered vestibular morphogenesis may be accompanied by altered mesenchymal functions. No significant difference could be appreciated between the normal and the homozygous mutant embryos at 10.5 and 11.5 dpc (data not shown). The distribution of proliferating cells in normal and mutant otocysts is summarized in the diagram in Fig. 6I. (red color).

The number and distribution of cells undergoing programmed cell death was evaluated by TUNEL staining of serial sections of 11.5-dpc otocysts. At this stage, clusters of apoptotic cells are found mainly in two regions: (1) at the base, tip, and along the endolymphatic duct; (2) and in a ventral–medial region of the otocyst (Fekete *et al.*, 1997; Nishizaki *et al.*, 1998; Nishikori *et al.*, 1999) (Figs. 7A and 7D). At later stages, apoptosis is observed in the central portion of the canal plates, in the chicken embryo. In the mouse embryo, the cells at the center of the canal plates seem to be recycled into the canal epithelium and do not show the hallmark of programmed cell death (Fekete *et al.*, 1997; Nishizaki *et al.*, 1998; Nishikori *et al.*, 1999).

Both the *Dlx5* and the *Nkx5.1* null embryos fail to develop proper semicircular canals, however with notable differences. In the *Nkx5.1* null otocyst, fusion plates are built but then the resorption of the central area is delayed and/or does not take place, while in the *Dlx5* null embryos, canal plates proper do not form. For these reasons, we have not investigated apoptotic events later than 11.5 dpc or associated with canal plate fusion.

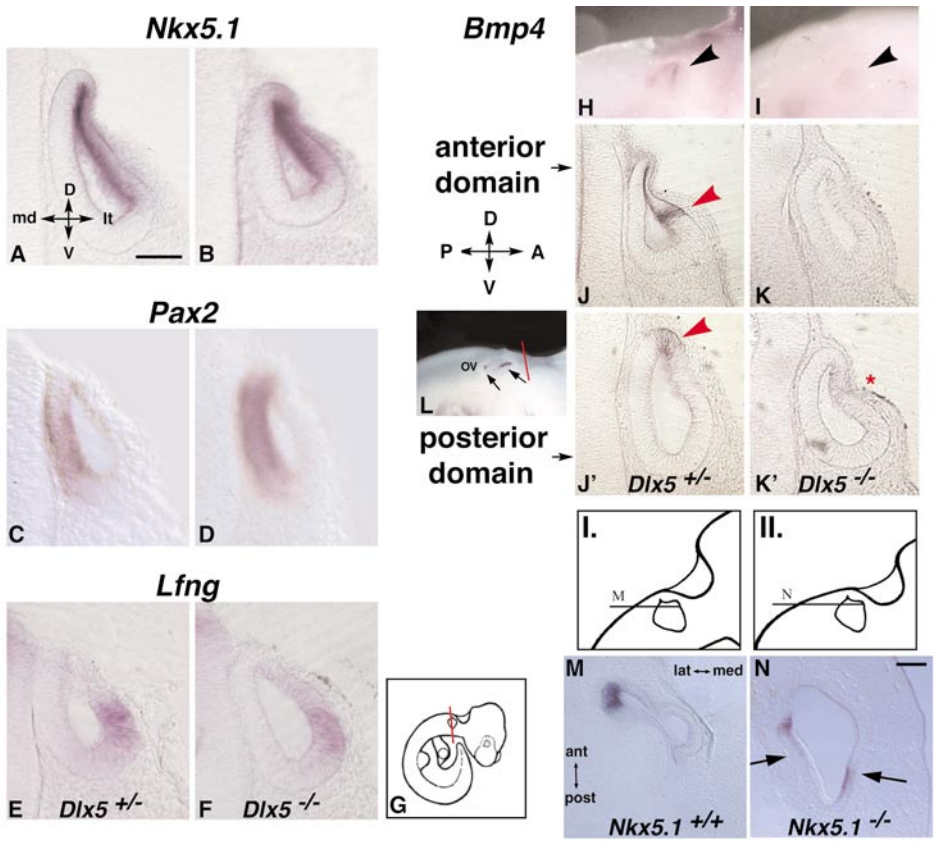
Serial sections of the otocyst of three normal and three homozygous *Dlx5* and *Nkx5.1* mutant embryos were compared. In the normal inner ear, TUNEL-positive cells were detected at the expected locations (Figs. 7A and 7D). In the *Dlx5* null embryos, at 11.5 dpc, apoptotic cells were seen in the expected regions, with a decreased number of positive cells along the endolymphatic duct (Figs. 7B and 7E, regions 1 and 2). The interesting finding is that novel clusters of apoptotic cells were seen, in particular, groups of TUNEL-positive cells were ectopically located in a posterior–lateral region (Fig. 7H, region 3) and in an anterior–medial region near the CVG. No significant difference in the number and distribution of apoptotic cells was observed in the primordium of the cochlea or in the CVG. In the *Nkx5.1* null embryos, TUNEL-positive cells were seen in slightly higher numbers compared with normal controls (Figs. 7C and 7F). The *Nkx5.1* null embryos also showed ectopic clusters of apoptotic cells, much like the *Dlx5* mutants: a posterior–lateral region (Fig. 7J, region 3) and an anterior–medial region near the CVG. Thus, an interesting similarity is observed between the *Dlx5* and the *Nkx5.1* mutant phenotypes. A summary of the distribution of apoptotic cells in the normal and mutant otocysts is reported (Fig. 7I.).

DISCUSSION

In this paper, we analyzed the cellular and molecular basis of inner ear defects caused by inactivation of the homeobox transcription factor *Dlx5*. The morphological malformations of the semicircular canals could be associated with changed patterns of apoptosis and proliferation along with striking reduction of *Bmp4* expression within the developing otocyst. These observations lead us to propose a fundamental role of *Dlx5* in the positional control of proliferation and apoptosis in the inner ear via regulation of *Bmp4* expression.

Morphogenesis of the semicircular canals begins around 11.5 dpc, and entails processes such as canal pocket outgrowth, canal plate formation, and fusion of the central region (Martin and Swanson ,1993). Such a complex process is far from being understood at cellular and molecular levels, but ultimately will have to be explained in terms of simple cellular events such as proliferation, migration, adhesion, and programmed death. Considerable efforts have been devoted to detecting and precisely mapping cell proliferation and apoptosis in the developing inner ear (Fekete *et al.*, 1997), in particular of the chicken embryo (Lang *et al.*, 2000), in which canal formation is the result of the remodeling of the canal plate due to cell proliferation (along the edges) and programmed cell death (at the center of the plate). Cell proliferation is clustered on the edges of the canal pockets, in the tip of the endolymphatic duct, and in areas of the inner ear undergoing elongation or growth, such as the edges of the forming semicircular canals. Proliferation of the epithelial cells of the developing inner ear is probably dependent on the presence of growth factors, such as fibroblast growth factor, insulin-like growth factors, and bombesin (Zheng *et al.*, 1997; Represa *et al.*, 1988). No data on BMP4 activity are available. Conversely, a tight control over the number and position of the apoptotic cells, and the time of onset, is essential for proper morphogenesis and is genetically imposed. In the mouse otocyst, apoptotic events can be detected at 11.0–11.5 dpc, before canal plate formation, at the base and tip of the developing endolymphatic duct and in a region on the ventral–medial surface of the otocyst. At slightly later stages, apoptosis is confined to sites where the otic epithelium makes obvious constrictions or bends. In the *Dlx5* null otocysts, the distribution of both apoptotic and proliferating cells is altered, implying a loss of positional control.

Dlx5 expression in the otocyst is initially found in region of the presumptive vestibulum and subsequently is confined to the epithelium of the developing semicircular canals, while it is absent in the utricle, saccule, or the cochlea. A number of transcription factors are expressed in specific territories of the otic vesicle (Torres and Giraldez, 1998) and may be components of a transcriptional cascade involving the *Dlx* genes. Such functional relationship has been ruled out in the case of the *Nkx5.1* and *Dlx5* homeobox genes, whose disruption leads to similar vestibular defects in the mouse (Hadrys *et al.*, 1998, Wang *et al.*, 1998; Acampora *et al.*, 1999; Depew *et al.*, 1999; this paper) for the following reasons: (1) *in situ* hybridization with *Dlx5* or *Nkx5.1* probes on, respectively, *Nkx5.1* and *Dlx5* null embryos did not show any difference between normal and mutant (Acampora *et al.*, 1999); and (2) these two genes respond in opposite ways to FGFs, known inner ear morphogens (Adamska *et al.*, 2001). We have also shown that the *Dlx5/LacZ*-expressing cell lineage persists in the *Dlx5* null embryos and shows normal pattern. Similarly, expression of general otocyst markers, such as *Pax2*, *Nkx5.1*,



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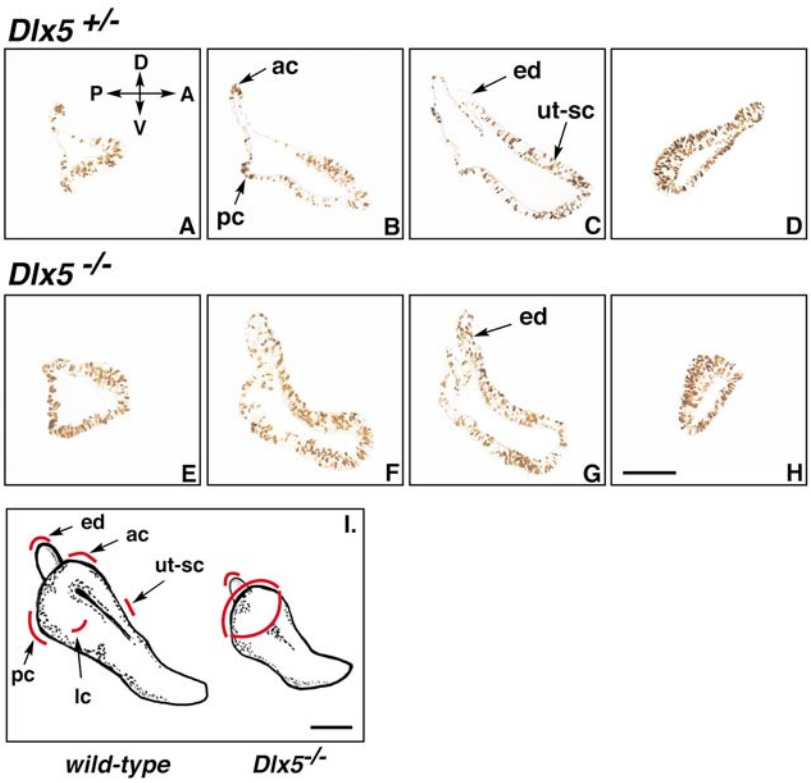

6

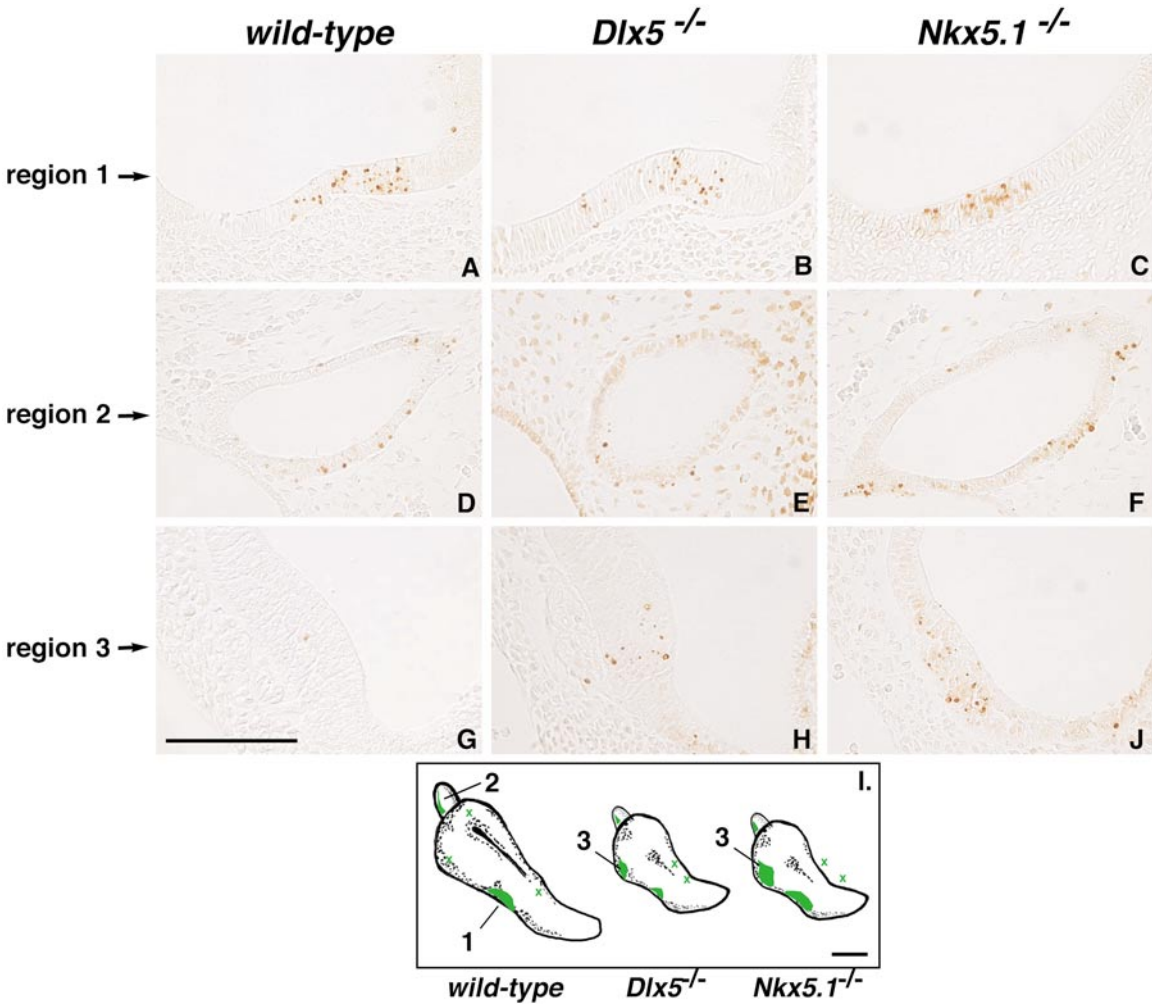

FIG. 7. Apoptosis in the otocyst *Dlx5*$^{-/-}$ embryos. Normal (A, D, G), *Dlx5*$^{-/-}$ (B, E, H), and *Nkx5.1*$^{-/-}$ (C, F, J) embryos at 11.5 dpc, in serial longitudinal sections. Same magnification is shown. (I) Diagram summarizing the location of the three main clusters (regions 1–3) of TUNEL-positive cells (green areas and crosses) in normal (left), *Dlx5*$^{-/-}$ (center), and *Nkx5.1*$^{-/-}$ (right) embryos. Regions 1, 2, and 3 correspond, respectively, to a central area of the inner ear at the base of the prospective cochlea, the endolymphatic duct, and a region of the vestibulum precursor of the semicircular canals. Scale bars (G, I.), 100 μm.

FIG. 5. Whole-mount *in situ* hybridization on *Dlx5*$^{-/-}$ embryos with otocyst markers. Embryos at 10.5 dpc were hybridized with the indicated probes and then sectioned, as illustrated (G, L: red lines). Orientation for sections (A)–(K′) is indicated in (A). (A–F) Hybridization with probes for *Nkx5.1* (A, B), *Pax2* (C, D), and *Lfng* (E, F). Normal (+/−) embryos are shown on the left (A, C, E); *Dlx5*$^{-/-}$ littermates are shown on the right (B, D, F). No significant differences were noted with these probes. (H–K′) Hybridization for *Bmp4* on *Dlx5*$^{-/-}$ embryos. Whole-mount hybridization (H, I) and frontal sections from these (J–K′) are shown. *Bmp4* is expressed in an anterior and a posterior domain (J, J′: red arrows), as shown by hybridization of a normal embryo (L). The orientation for (L) is shown above it. The section plane corresponding to pictures (J)–(K′) is shown (L, red line). Two sections for each of the normal (J, J′) and the mutant (K, K′) otocysts are shown, corresponding to the anterior (J, K) and posterior (J′, K′) *Bmp4* expression domain (red arrowheads). Note the nearly complete absence of *Bmp4* signal in the *Dlx5*$^{-/-}$ (red asterisk indicates a faint *Bmp4* signal seen only in one mutant embryo). (M, N) Hybridization on normal (M) and *Nkx5.1*$^{-/-}$ (N) embryos for *Bmp4*. In the *Nkx5.1*$^{-/-}$ embryo, *Bmp4* signal is much reduced and mispositioned. The orientation is indicated in (M); the section planes are indicate in (I) and (II). ant, anterior; post, posterior; lat, lateral; med, median. Black arrows in (F)–(J) point to regions of ectopic expression. lt, lateral; md, medial; OV, otic vesicle; A, anterior; D, dorsal; P, posterior; V, ventral. Scale bars (A, N), 100 μm.

FIG. 6. Cell proliferation in the otocyst of *Dlx5*$^{-/-}$ embryos. Staining with anti-BrdU antibody of normal (A–D) and *Dlx5*$^{-/-}$ (E–H) embryos at 11.5 dpc in serial longitudinal sections. (I) Schematic diagram summarizing the location of higher density BrdU-positive nuclei (red lines) in normal (left) and *Dlx5*$^{-/-}$ (right) embryos. Abbreviations as in previous figures. Scale bars (H, I), 100 μm.

Otx2, and *Lfng*, is preserved in $Dlx5^{-/-}$ otocysts. Thus, the severe vestibular defects observed in the *Dlx5* mutant animals are not likely to reflect a more general alteration of the otocyst patterning, nor do they seem to involve changes in expression of the above genes. Rather, some properties of the vestibular epithelium, such as expression of a morphogen, must be affected in the *Dlx5* mutants and lead to impaired canal morphogenesis.

The altered distribution of proliferating and TUNEL-positive cells compared with the normal controls is well compatible with the lacking or altered distribution of a diffusable molecule. Noteworthy, the morphogenetic defects in the *Dlx5* null inner ear are not restricted to the semicircular canals but extend to other inner ear regions, where *Dlx5* is not expressed, such as the cochlea. Thus, the disruption of *Dlx5* exerts some indirect, nonautonomous effects on developing inner ear structures. Such long-distance influence is also suggested by the observation that the peri-otic mesenchyme of *Dlx5* mutants fails to express the transcription factor *goosecoid*, although *Dlx5* is not expressed in this tissue (Depew *et al.*, 1999). This strongly implies that expression of *goosecoid* is regulated via a *Dlx5*-dependent diffusable molecule, secreted by the *Dlx5*-expressing vestibular epithelium. Further support for the existence of a *Dlx5*-dependent morphogen in the inner ear development is provided by analysis of the sensory component of *Dlx5* mutant inner ear, presented in this paper. In the *Dlx5* mutant mice, the *cristae* are nearly completely absent, while a single patch of calretinin-positive cells is present in a position roughly corresponding to the *crista* of the posterior canal. The *maculae* are present but the calretinin-immunoreactive cells appear sparse and disorganized. Finally ectopically positioned calretinin-positive cells have been noticed, in regions not expressing *Dlx5* mRNA. Clearly, the lack of *Dlx5* does not, per se, abolish the ability of the sensory lineage to differentiate. In this regard, other studies have shown that sensory patches, with fully differentiated hair cells, can form even in grossly malformed inner ears (Fekete, 1996; Hadrys *et al.*, 1998; Wang *et al.*, 1998; Salminen *et al.*, 2000). Therefore, in *Dlx5* null mice, subregionally altered positional cell identity might lead to a misplacement of differentiating cells, a rather random distribution of proliferating cells, and the abnormal clustering of apoptotic cells.

Altered localization of proliferation, differentiation, and apoptosis is accompanied by a severe down-regulation of *Bmp4* expression. In view of the above considerations and of the lack of *Bmp4* expression in the *Dlx5* null embryos, it is tempting to speculate that *Bmp4* represents a key factor for normal inner ear morphogenesis, and that *Bmp4* imposes a precise regionalization of the proliferative, apoptotic, and differentiation events. Strong support for this notion comes from experiments conducted on the chicken embryos, in which *noggin* beads were implanted in the otic region of the head, in order to sequester endogenous BMP4. The manipulated embryos showed varying degrees of semicircular canal and vestibular dysmorphogenesis (Chang *et al.*, 1999; Gerlach *et al.*, 2000), much like the phenotype of the *Dlx5* and *Nkx5.1* knock-outs. Regrettably, mice knock-out for *Bmp4* die *in utero* very early and cannot be analyzed for inner ear development (Winnier *et al.*, 1995). Conclusive evidence on the role of BMP4 in the morphogenesis of the mammalian inner ear can only be achieved with the generation of conditional *Bmp4* null mice and the use of specific inner ear promoter–*CRE* constructs. An apoptotic controlling function of *Bmp4* is not necessarily in contrast with its apoptosis-promoting action observed in other systems. The relationship between *Bmp4* expression and apoptosis is not straightforward: the increase in apoptosis is more pronounced in the *Nkx5.1* mutant, while *Bmp4* is more dramatically down-regulated in the *Dlx5* mutant. Further experiments are necessary to evaluate the exact role of BMP4 on apoptotic processes in the developing inner ear.

Too little is known about the regulation of *Bmp4* expression within developing otic epithelium. We show for the first time that two independently acting homeobox genes, *Dlx5* and *Nkx5.1*, both of which play a role in the morphogenesis of the vestibular organ and whose inactivation leads to similar, but not identical defects, appear to be necessary for correct *Bmp4* expression in the otocyst and for regional control on apoptosis. It is not known whether the *Dlx5* and *Nkx5.1* homeogenes directly regulate the *Bmp4* promoter. The fact that *Bmp4* is expressed by groups of epithelial cells residing within the *Dlx5*- and the *Nkx5.1*-expressing epithelial domain of the otocyst suggest that both genes may well act cell autonomously on *Bmp4* expression.

Mice with a targeted disruption of the *Dlx5* gene have been shown to suffer severe craniofacial defects, including dysmorphogenesis of the otic capsule of the temporal region of the head (Acampora *et al.*, 1999; Depew *et al.*, 1999). This defect cannot be attributed to an early function of *Dlx5* in the peri-otic mesenchyme, since *Dlx5* is not expressed in this tissue until much later in development, at the time of bone tissue differentiation (Acampora *et al.*, 1999; G.R.M., unpublished observations). Furthermore, morphogenetic defects affecting the otocyst are seen much earlier in time (11.5 dpc) than the appearance of any differentiating cartilage tissue. Thus, dysmorphogenesis of the otic cartilage capsule is likely due to indirect effects of the *Dlx5* disruption. In spite of this, epithelial–mesenchymal interactions are certainly affected in the *Dlx5* null animals and may explain some aspects of the inner ear phenotype. Depew *et al.* (1999) have reported the lack of expression of the transcription factor *goosecoid* in the otic and nasal mesenchyme of *Dlx5* null mice, indicating that peri-otic mesenchyme is affected in these mice. Interestingly, the compound knock-out of two *aristaless*-related homeogenes *Prx1* and *Prx2*, expressed in the peri-otic mesenchyme, leads to vestibular malformation and the lack of anterior and posterior semicircular canals, indicating the requirement for proper mesenchyme in the morphogenesis of the inner ear canals (ten Berge *et al.*, 1998). Thus, impaired morphogenesis of *Dlx5* null otocyst may also be due to mesenchymal defects. We are now able to draw an exciting

parallel between olfactory and otic placode development in the mouse embryo: both are sites of *Dlx5* expression, both are affected by severe dysmorphogenesis and hypoplasia in the null mice (Acampora *et al.*, 1999; Depew *et al.*, 1999), and the epithelial cells of both systems express *Bmp4* mRNA (Shou *et al.*, 2000; Morsli *et al.*, 1999), which may act as a morphogen, organizer, or survival factor.

To summarize the hypotheses that could be derived from our results: the severe dysmorphogenesis of the semicircular canals observed in $Dlx5^{-/-}$ embryos is associated with a general loss of positional control of basic cellular events, such as proliferation, differentiation, and apoptosis. This phenotype is compatible with an alteration in the expression and/or distribution of a *Dlx5*-dependent diffusable inner ear morphogen. *Dlx5* and other homeobox genes may control inner ear morphogenesis through tight regulation of expression and localization of *Bmp4* mRNA, which in turn may be necessary for regionalization within the otic epithelium.

ACKNOWLEDGMENTS

We thank Drs. D. Wu (NIDCD, Bethesda, MD) and A. Simeone (King's College, London, UK) for generous gift of probes. We thank Dr. O. Barbieri for her help in the animal work and Dr. F. Cecconi (RDMTI University "Tor Vergata," Rome, Italy) for helpful criticism and for sharing unpublished data. G.R.M. is recipient of a Telethon–Italy Career Award (03/cp). G.L. is supported by Telethon–Italy (Project GP0218/01), FISM (2000/R/41), CNR (Progetto Finalizzato Biotecnologie), and Ministero della Sanità. L.P. is a recipient of a fellowship from AIRC, Italy. M.A. is a fellow of the Boehringher–Ingelheim Fonds.

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Received for publication February 22, 2002
Revised April 22, 2002
Accepted April 24, 2002
Published online June 14, 2002